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11/19/03 Esther Marques
Date Esther Marques

Docket No.	CACE/0003L

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TITLE OF THE INVENTION (280 characters max)

METHODS FOR PRODUCING BLOOD PRODUCTS

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ENCLOSED APPLICATION PARTS (*check all that apply*)

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UNITED STATES PROVISIONAL PATENT APPLICATION FOR:

METHODS FOR PRODUCING BLOOD PRODUCTS

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METHODS FOR PRODUCING BLOOD PRODUCTS

[0001] **FIELD OF THE INVENTION**

[0002] This invention relates generally to the in vitro production of clinically useful quantities of mature blood cells and blood products from human embryonic stem cells.

[0003] **BACKGROUND OF THE INVENTION**

[0004] The availability of transfusable blood products is decreasing every year despite advances in blood typing and screening for the presence of infectious disease. In some countries, this lack of availability is at a crisis. The high incidence of blood borne diseases such as HIV and hepatitis severely limits the number of available donors and increases the risk of contracting an infection from a heterologous blood transfusion. Moreover, even with typing and cross-matching, there continue to be risks associated with blood transfusion including febrile or urticarial reactions and non-fatal or fatal hemolytic reactions.

[0005] Human embryonic stem cells (hESCs) are derived from the inner cell mass of a blastocyst-stage human embryo, and are capable of undergoing an unlimited number of cell divisions without differentiating while maintaining a stable, diploid complement of chromosomes. hESCs are pluripotent and, under various culture regimes, can give rise to any of the differentiated cell types derived from the three primary germ layers of the embryo (the endoderm, mesoderm and ectoderm). The path of differentiation from an hESC to a fully-differentiated cell involves a series of steps resulting in a series of cell intermediates. As the differentiation

process advances, it leads to a progressive diminution of the differentiation potential of each resulting cell.

[0006] hESCs can, under certain culture conditions and through a series of intermediates, give rise to mature hematopoietic cells. Mature hematopoietic cells comprise lymphocytes (B and T cells of the immune system) and myeloid cells (erythrocytes, megakaryocytes, granulocytes and macrophages). The lymphoid lineage, comprising B cells and T cells, provides for the production of antibodies, regulation of the cellular immune system, and detection of agents foreign to the host. The myeloid lineage, which includes monocytes, granulocytes, megakaryocytes as well as other cells, monitors for the presence of foreign bodies, provides protection against neoplastic cells, scavenges foreign materials, and produces platelets. The erythrocyte lineage provides red blood cells, which act as oxygen carriers.

[0007] There are many uses for blood cells and blood products. Platelets find use in protection against hemorrhage as well as provide a source of platelet derived growth factor. Red blood cells find use in transfusions to support the transport of oxygen. Specific lymphocytes find application in the treatment of various diseases, for example, where the lymphocyte is specifically sensitized to an epitope of an antigen. Blood products also may be used for rescue from high dose cancer chemotherapy or for many other purposes.

[0008] Several attempts have been made to address the need for transfusable blood products. For example, native hemoglobin has been chemically modified by various methods in an attempt to create a blood substitute, but thus far such products suffer from a variety of shortcomings, including nephrotoxicity, excessive O_2 affinity, a short half-life, rapid dimerization and excretion, and insufficient plasma concentration (see, e.g., Skolnick, J. Amer. Med. Assoc., 268:697 (1992); Vigerou et al., Bull. Acad. Natl. Med., 174:947 (1990)). Alternatively, human hemoglobin has been packaged in liposomes for administration as neo-erythrocytes, but such products are difficult to sterilize (particularly against viruses such as HIV), exhibit a short half-life because they are rapidly cleared by the reticuloendothelial system, and suppress the immune system significantly, thereby predisposing recipients to an increased infection rate (Djordjerich et al, Crit. Rev. Ther. Carrier Syst., 6:131

(1989)). In addition, perfluorochemicals have been tested as hemoglobin substitutes, but these perfluorocarbons contain a potentially toxic surfactant (Pluronic F-68), they must be stored frozen, and, due to their insolubility, require emulsification. Moreover, these fluids require oxygen-enriched air for proper oxygen delivery, as well as frequent administration due to a short half-life.

[0009] Despite these efforts, an effective and safe blood substitute is still not available. Thus, there is a desire in the art for the in vitro production of clinically useful quantities of mature blood cells and blood products.

SUMMARY OF THE INVENTION

[0010] The present invention provides methods and apparatus to produce clinically useful amounts of natural, mature, differentiated, universally-compatible, or, in some instances, specifically-engineered human blood cells and blood products under conditions such that the major risks from blood-borne infectious agents and transfusion reactions are absent. Immortal pluripotent cells (*e.g.*, hESCs) are cultured in the presence of combinations of maintenance-, proliferation- and growth- and/or maturation-promoting factors, such as cytokines, lymphokines, colony stimulating factors, mitogens, growth factors, and/or other maturation factors, so as to produce at will clinically useful quantities of a single species of infectious agent-free human blood cells such as erythrocytes, lymphocytes, megakaryocytes, monocytes, macrophages, neutrophils, eosinophils, basophils, plasma, platelets, as well as expanded stem cell cultures. The immortal pluripotent cells to be cultured are preferably blood group type O, and Rh factor negative ("universal donor cells").

[0011] In general, it is one object of this invention to provide a hematopoietic cell production device comprising a sequential series of bioreactors and selection systems, This cell production device will provide a method to: 1) culture immortal pluripotent cells (*e.g.*, hESCs) 2) proliferate transit amplifying (TA) cells, and 3) differentiate TA cells into increasingly-differentiated TA cells and, ultimately, into fully differentiated mature cells from the hematopoietic lineage. The cell production

device is designed to produce clinically relevant quantities of blood or blood products, e.g., on a multi-liter scale.

[0012] In a specific embodiment, cells collected in a final step of the system are in a similar concentration as found in native blood. In another specific embodiment, cells collected in a final step are in a higher concentration than native blood. In either case, the cells in the final step can be provided in pharmaceutically acceptable solution. The cells in the pharmaceutically acceptable carrier are a therapeutic product for delivery via transfusion of the cells to the circulatory system of a patient.

[0013] For example, it is one object of this invention to provide a sequential series of bioreactors and selection systems for producing in clinically useful quantities of universal-donor erythrocytes from universal-donor hESCs for transfusion into, e.g., anemic or thrombocytopenic patients. In other aspects of the invention, different apparatus and methods will be used to generate different species of mature cells of the hematopoietic lineage.

[0014] An important aspect of the invention is a starting "culture" of immortal cells that are self renewable over a span of time, preferably at least three months. More preferably at least six months, but cells that are renewable for one year or longer are most preferable for use as a starting culture. The current invention is described throughout as using hESCs as the starting cell population, but it is also envisaged that other immortal pluripotent cell populations (*e.g.*, subpopulations of hESC that are optimized for hematopoietic differentiation, modified HSC de-differentiated to allow immortalization in culture) could be used. Thus, although the described embodiments use hESCs as the exemplary immortal pluripotent, the invention is also intended to include other immortal cell populations.

[0015] It is yet another object of the invention to provide a method for the production of genetically-modified immortal pluripotent cells so as to eliminate cell surface antigens, such as human histocompatibility antigens and blood group antigens. These genetically-modified immortal pluripotent cells can then be

expanded and differentiated to produce, for example, single species of erythrocytes, platelets, leukocytes and other mature blood cells for transfusion purposes.

[0016] Thus, the present invention provides methods, devices and apparatus to produce blood products in vitro comprising culturing immortal pluripotent cells in a culture bioreactor to produce daughter pluripotent cells and TA cells; removing the TA cells from the culture reactor; proliferating the TA cells in a proliferation reactor; and differentiating the TA cells in a differentiation reactor to produce a homogeneous population of mature blood cells. In addition, one or more selection or filtration steps may be performed after the culturing step, after the removing step, after the proliferating step and/or after the differentiation step. Also, the method may include one to many additional differentiation steps in sequence or alternating with one or more selection steps. Moreover, a preservation and/or packaging step may be performed after the differentiating step.

[0017] These and other objects, advantages and features of the present invention will become apparent to those persons skilled in the art upon reading the details of the structure of the device, formulation of compositions and methods of use, as more fully set forth below..

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] So that the manner in which the above recited features, advantages and objects of the present invention are attained and can be understood in detail, a more particular description of the invention, briefly summarized above, may be had by reference to the embodiments that are illustrated in the appended drawings. It is to be noted, however, that the appended drawings illustrate only certain embodiments of this invention and are therefore not to be considered limiting of its scope, for the present invention may admit to other equally effective embodiments.

[0019] Figure 1 is a flow chart showing the steps of a method according to one embodiment of the present invention.

[0020] Figure 2A shows a dividing scheme for hESCs that provides a steady state population of hESCs while generating TA cells (cells in the process of differentiation). Figure 2B shows a dividing scheme for hESCs that provides for the expansion or amplification of an hESC population.

[0021] Figure 3 is a diagram showing immune and blood cells at different stages of development, from an hESC at the left to cells of increasing differentiation moving to the right.

[0022] **DETAILED DESCRIPTION**

[0023] Before the present devices, cells and methods of cell production are described, it is to be understood that this invention is not limited to the particular methodology, products, apparatus and factors described, as such methods, apparatus and formulations may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by appended claims.

[0024] It must be noted that as used herein and in the appended claims, the singular forms "a," "and," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a factor" refers to one or mixtures of factors, and reference to "the method of production" includes reference to equivalent steps and methods known to those skilled in the art, and so forth.

[0025] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing devices, formulations and methodologies which are described in the publication and which might be used in connection with the presently described invention.

[0026] In the following description, numerous specific details are set forth to provide a more thorough understanding of the present invention. However, it will be

apparent to one of skill in the art that the present invention may be practiced without one or more of these specific details. In other instances, well-known features and procedures well known to those skilled in the art have not been described in order to avoid obscuring the invention. For example, additional description of apparatus, methods, cell populations and appropriate factors that could be employed for the methods of expansion and differentiation described herein include those described in U.S. Pat Nos. 5,399,493; 5,472,867; 5,635,386; 5,635,388; 5,646,043; 5,674,750; 5,925,567; 6,403,559; 6,455,306; 6,258,597; and 6,280,718.

[0027] Generally, conventional methods of cell culture, stem cell biology, and recombinant DNA techniques within the skill of the art are employed in the present invention. Such techniques are explained fully in the literature, see, e.g., Maniatis, Fritsch & Sambrook, Molecular Cloning: A Laboratory Manual (1982); DNA Cloning: A Practical Approach, Volumes I and II (D.N. Glover, ed. 1985); Animal Cell Culture (R.I. Freshney, ed. 1986); and Embryonic Stem Cells, (Kurstad Turksen, Ed., Humana Press, 2002).

[0028] The term "blood cells" is intended to include erythrocytes (red blood cells), reticulocytes, megakaryocytes, eosinophils, neutrophils, basophils, platelets, monocytes, macrophages, granulocytes and cells of the lymphoid lineage. For the purpose of transfusion into patients, erythrocytes, granulocytes and platelets are particularly valuable. The phrase "clinically useful quantities (or amounts) of blood cells" is intended to mean quantities of blood cells of whatever type sufficient for transfusion into human patients to treat a clinical condition. In addition, the term "transit amplifying cell" or "TA cell" refers to an intermediately differentiated cell--that is, a cell more differentiated than the initial immortal pluripotent cell, yet less differentiated than mature cells of the hematopoietic lineage such as those listed above.

[0029] Figure 1 is a flow chart showing generally the steps of one method (100) according to one embodiment of the present invention. In the first step of method 100, step 110, hESCs are obtained and cultured in a first bioreactor.

[0030] Procurement of hESCs

[0031] Human embryonic stem cells (hESCs) may be derived from the inner cell mass of a blastocyst stage human embryo or an established cell line may be used (such as those developed by Thomson and Odorico, Trends Biotechnol., 18:53-57 (2002), namely, H1, H7, H9.1, H9.2, H13 or H14). To generate human ES cell cultures de novo, cells from the inner cell mass are separated from the surrounding trophectoderm by microsurgery or by immunosurgery (which employ antibodies against the trophectoderm that break it down) and are plated in culture dishes containing growth medium supplemented with fetal bovine serum (alternatively, KnockOut Dulbecco's modified minimal essential medium containing basic FGF can be used without serum), usually on feeder layers of mouse embryonic fibroblasts that have been gamma-irradiated to prevent replication. Alternatively, a feeder-free culture system may be employed, such as that reported by Chunhui Xu, Melissa Carpenter and colleagues using Matrigel or laminin as a substrate, basic FGF, and conditioned medium from cultures of mouse embryo fibroblasts (Xu, et al., Keystone Symposia. *Pluripotent stem cell biology and applications: Growth of undifferentiated human embryonic stem cells on defined matrices with conditional medium.* Poster abstract 133).

[0032] Once the hESC culture has been established, it can be placed within the first bioreactor (the culture bioreactor) of the present invention for growth, although an optional selection step may be performed before transferring the initial hESC culture into the bioreactor.

[0033] Selection at this juncture and in other steps in the methods according to the present invention can be performed in any way known in the art. The most robust selection method for hESCs and various TA intermediates to date employs the use of cell surface markers specific to a desired cell type (or cell surface markers specific to an undesired cell type when employing negative selection). Cell surface markers are specialized proteins, usually receptors, which have the capability of selectively binding or adhering to other signaling molecules. Cell surface markers

differ markedly in their structure and their affinity for ligands. In many cases, a combination of multiple markers is used to identify a particular cell type. The cell surface markers are exploited for selection by using, e.g., fluorescent labeling and fluorescent activated cell sorting (FACS) where the ligand of a specific receptor or a group of ligands is fluorescently labeled and allowed to bind to a cell population. The cells are subjected to light excitation at a wavelength appropriate for the fluorophore, and then sorted by virtue of their fluorescent profile. A cell surface marker useful for selection of hESC is CD30. In addition, other techniques can be used to identify hESCs, such as techniques to detect hESC cell products such as SSEA-4, TRA-1-60, and GCM-2.

[0034] Alternatively, ligands to the cell surface markers can be immobilized to a surface of a culture flask, bead, or other surface (such as the surface of a bioreactor), and the cells to be sorted are exposed to the ligand/immobilizing surface. Cells that have cell surface receptors to the ligand will bind the ligand becoming immobilized, where cells lacking the cell surface receptor to the ligand will not bind and can eluted or otherwise separated from the bound cells.

[0035] Any ligand that is specific for hESCs may be used in order to obtain a homogenous population of hESCs for use in the methods of the present invention. For example, such a ligand may be an antibody to a cell surface marker. In one embodiment of the invention, the ligand is a monoclonal antibody to cell surface marker CD30 (cluster designation 30), a molecule found specifically on hESCs. Other ligands include any agents that preferentially bind to specific hESC surface markers. Alternatively, negative selection may be employed where undesired cell populations (lineage committed cell, e.g.) are removed from the population of hESCs.

[0036] Once hESCs are obtained for culture, they are placed in the first bioreactor of the present invention, the culture bioreactor.

[0037] Bioreactors

[0038] The bioreactors used at the various steps of the present invention are designed to provide a culture process that can deliver medium and oxygenation at

controlled concentrations and rates that mimic nutrient concentrations and rates in vivo. Bioreactors have been available commercially for many years and employ a variety of types of culture technologies. Of the different bioreactors used for mammalian cell culture, most have been designed to allow for the production of high density cultures of a single cell type and as such find use in the present invention. Typical application of these high density systems is to produce as the end-product, a conditioned medium produced by the cells. This is the case, for example, with hybridoma production of monoclonal antibodies and with packaging cell lines for viral vector production. However, these applications differ from applications where the therapeutic end-product is the harvested cells themselves as in the present invention.

[0039] Once operational, bioreactors provide automatically regulated medium flow, oxygen delivery, and temperature and pH controls, and they generally allow for production of large numbers of cells. Bioreactors thus provide economies of labor and minimization of the potential for mid-process contamination, and the most sophisticated bioreactors allow for set-up, growth, selection and harvest procedures that involve minimal manual labor requirements and open processing steps. Such bioreactors optimally are designed for use with a homogeneous cell mixture as contemplated by the present invention. Suitable bioreactors for use in the present invention include but are not limited to those described in US Pat. No. 5,763,194 to Slowiaczek, et al., particularly for use as the culture bioreactor; and those described in US Pat. Nos. 5,985,653 and 6,238,908 to Armstrong, et al., US Pat. No. 5,512,480 to Sandstrom, et al., and US Pat. Nos. 5,459,069, 5,763,266, 5,888,807 and 5,688,687 to Palsson, et al., particularly for use as the proliferation and differentiation bioreactors of the present invention.

[0040] The culture system in one aspect according to the present invention consists of a variable number of bioreactors connected to differing medium sources by sterile tubing and to one another with, in some embodiments, intervening selection apparatus. Generally, the medium is circulated through the bioreactor with the aid of a roller or pump. The bioreactors optimally include probes to measure pH, temperature, and O_2 concentration at points before and following each bioreactor(s).

Information from these sensors may be monitored electronically. In addition, provision may be made for obtaining serial samples of the growth medium in order to monitor waste or electrolyte concentration, as well as proliferation and differentiation factor and nutrient concentrations. Activities of proliferation and differentiation factor samples taken from the entry and/or exit points of the bioreactors may be measured by conventional bioassays or immunoassays.

[0041] When an appropriate endpoint has been obtained in any one bioreactor (whatever the appropriate endpoint might be for that bioreactor), the cells are transferred out of the bioreactor, and most likely are fed directly into a selection apparatus or other bioreactor. Selection apparatus are known in the art, and include FACS instrumentation and other fluorescent detection devices, immunologic methodologies, binding/immobilization assays and the like.

[0042] With any large volume cell culture, several fundamental parameters require almost constant control. Cultures must be provided with the medium that allows for, in the present invention, hESC maintenance, TA cell proliferation, TA cell differentiation--perhaps several separate differentiation cultures and conditions--as well as final cell culture/preservation. Typically the various media are delivered to the cell by a pumping mechanism in the bioreactor, feeding and exchanging the medium on a regular basis. The exchange process allows for by-products to be removed from the culture. Growing cells or tissue also requires a source of oxygen. Different cell types have different oxygen requirements, and the differing cell cultures of the present invention may have differing oxygen delivery requirements depending on the density of the culture. Accordingly, a flexible and adjustable means for providing oxygen to the cells is a desired component.

[0043] Depending on the particular culture, even distribution of the cell population and medium supply in the culture chamber can be an important process control. Such control is often achieved by use of a suspension culture design, which can be effective where cell-to-cell interactions are not important. Examples of suspension culture systems include various tank reactor designs and gas-permeable plastic bags. For cells that do not require assembly into a three-dimensional structure or require proximity to a stromal or feeder layer--such as most blood cell precursors or

mature blood cells--such suspension designs may be used.

[0044] Efficient collection of the cells at the completion of the culture process is an important feature of an effective cell culture system. One approach for production of cells as a product is to culture the cells in a defined space, without physical barriers to recovery, so that simple elution of the cell product results in a manageable, concentrated volume of cells amenable to final washing in a commercial, closed system cell washer designed for the purpose. Optimally, the system would allow for addition of a pharmaceutically acceptable carrier, with or without preservative, or a cell storage compound, as well as provide efficient harvesting into appropriate sterile packaging. Optimally the harvest and packaging process may be completed without breaking the sterile barrier of the fluid path of the culture chamber.

[0045] With any cell culture procedure, a major concern is sterility. When the product cells are to be transplanted into patients--often at a time when the patient is ill or immunocompromised--absence of microorganisms is mandated. An advantage of the present cell production device over manual processes is that, as with many described bioreactor systems, once the culture is initiated, the culture chamber and the fluid pathway is maintained in a sterile, closed environment.

[0046] Culturing Bioreactor Conditions

[0047] When hESCs divide, some if not all the divisions are asymmetric. Such a scheme is shown in Figure 2A. In asymmetric division, an initial hESC (10), divides to produce a daughter hESC (20) and a TA cell (30). Asymmetric division leads to a steady state hESC population, generating a population of TA cells to be used for further differentiation in methods according to the present invention. Figure 2B shows hESC amplification where the initial hESC divides to produce two daughter cells (20). In this scheme, no TA cell is generated, and the hESC population grows logarithmically. To date, there is evidence that hESCs divide some of the time, if not most of the time, asymmetrically. The present invention exploits the asymmetric process in the culturing bioreactor at step 110, where hESCs are cultured and maintained in a steady state with each hESC dividing to produce one hESC and one

TA cell and where TA cells are removed from the culture bioreactor to continue through the process steps of the present invention.

[0048] The bioreactor used for step 110 is a bioreactor with a "smart surface" such as that disclosed in US Pat. No. 5,763,194 to Slowiaczek, et al. Herein, the phrase "smart surface" refers to a surface in a bioreactor that has been modified to comprise a ligand that binds differentially to a certain cell type. In one aspect of the present invention, the ligand is specific for hESCs, and will not bind TA cells. One such ligand is the CD30 ligand that was suggested for use for screening the cultured hESCs before transfer to the bioreactor at step 110. Because the TA cells do not bind to the smart surface, they can be removed from the culturing bioreactor and, thus, separated from the hESC culture (step 112). In the case of hESCs, the bioreactor chosen for the culturing bioreactor may have to be configured to support growth of a feeder layer in contact or in close proximity to the hESCs. Alternatively, should a feeder-free culture be desired, the bioreactor would not have to be so configured. At this point, a separate, optional selection step (step 114) may take place in addition to the selection that takes place by virtue of the smart surface. Selection technologies have been discussed herein and are known to those skilled in the art.

[0049] Proliferation Reactor Conditions

[0050] The bioreactor and culture conditions used to proliferate the TA cells vary depending on the ultimate mature cell product desired. The proliferating bioreactor does not necessarily require a smart surface (though one could be employed in various aspects of the invention). Several "classic" bioreactors are known in the art and may be used, including bioreactors as described in US Pat. Nos. 5,985,653 and 6,238,908 to Armstrong, et al., US Pat. No. 5,512,480 to Sandstrom, et al., and US Pat. Nos. 5,459,069, 5,763,266, 5,888,807 and 5,688,687 to Palsson, et al.

[0051] In general, proliferation conditions include various media. Illustrative media include Dulbecco's MEM, IMDM and RPMI-1640 that can be supplemented with a variety of different nutrients and growth factors. The media can be serum-free or supplemented with suitable amounts of autologous serum. One suitable medium

is one containing IMDM, effective amounts of at least one of a peptone, a protease inhibitor and a pituitary extract and effective amounts of at least one of human serum albumin or plasma protein fraction, heparin, a reducing agent, insulin, transferrin and ethanolamine. Other suitable media formulations are the SSP media disclosed in US Pat. No. 5,728,581 to Schwartz.

[0052] The proliferated TA cells from step 120 are then transferred to a third bioreactor, the differentiation bioreactor at step 122. This transfer step may or may not involve a selection/quality control step (step 124) where only TA cells of a certain phenotype are selected to go through to the next bioreactor for differentiation.

[0053] The TA cells following proliferation may be hematopoietic stem cells (HSC), hemangioblasts, or other uncommitted common precursors of hematopoietic cells. Hemangioblasts are stable, non-transient cells that are present in both newborn infants and adults and have been isolated from cord blood. Hemangioblasts can be proliferated in a separate proliferation step or passed from the proliferation reactor to the differentiation reactor, possibly with an intervening selection step 124. If hemangioblasts are desired, selection at step 124 should enrich for cells that are $CD34^-$, Lin^- or $CD34^-$, $Cd2^-$, $CD3^-$, $Cd14^-$, $CD16^-$, $CD24^-$, $CD56^-$, $CD66b^-$, glycophorin A^-, $flk1^+$, $CD45^+$, $CXCR4^+$, and MDR^+. Exemplary factors, methods, culture condition and the like for inducing differentiation of the hESC to hematopoietic precursor cells such as HSC include those disclosed in US Pat. No. 6,280,718 to Kaufman et al.

[0054] Alternatively or at a more differentiated state, the proliferated TA cells may exhibit characteristics of human hematopoietic stem cells, and, as such, it would be desirable to enrich the proliferation population for cells that are CD34+, CD59+, and/or Thy-1+ prior to differentiation, using for example, the method of Sutherland et al., Exp. Hematol., 20:590 (1992) or that described in U.S. Pat. No. 4,714,680 (at step 124 of Figure 1). In addition, it may be desirable to subject the proliferated cells to negative selection to remove those cells that express lineage markers. LIN- cells lack several markers associated with lineage committed cells. Lineage committed markers include those associated with T cells (such as CD2, 3, 4 and 8), B cells (such as CD10, 19 and 20), myeloid cells (such as CD14, 15, 16 and 33), natural

killer ("NK") cells (such as CD2, 16 and 56), RBC (such as glycophorin A), megakaryocytes (CD41), or other markers such as CD38, CD71, and HLA-DR. Populations highly enriched in stem cells and methods for obtaining them are described in PCT/US94/09760; PCT/US94/08574 and PCT/US94/10501.

[0055] Differentiation Bioreactor Conditions

[0056] Once the proliferated cells have been subjected to selection, they can be differentiated into a differentiated mixed cell population or a specific species of blood cell, selectively. The differentiation bioreactor may vary depending on the desired differentiated cell; however, for most applications, the differentiation bioreactor may be a "classic" bioreactor such as that suggested for the proliferating bioreactor and as described in US Pat. Nos. 5,985,653 and 6,238,908 to Armstrong, et al., US Pat. No. 5,512,480 to Sandstrom, et al., and US Pat. Nos. 5,459,069, 5,763,266, 5,888,807 and 5,688,687 to Palsson, et al.

[0057] Differentiation conditions, such as medium components, O_2 concentration, differentiation factors, pH, temperature, etc., as well as the bioreactor employed, will vary depending on the intermediate to be differentiated and the desired differentiated cell type, but will differ primarily in the cytokine(s) used to supplement the differentiation medium. Such cytokines will be used at a concentration from about 0.1 ng/mL to about 500 ng/mL, more usually 10 ng/mL to 100 ng/mL. Suitable cytokines include but are not limited to c-kit ligand (KL) (also called steel factor (Stl), mast cell growth factor (MGF), and stem cell growth factor (SCGF)), macrophage colony stimulating factor (MCSF), IL-1 α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, G-CSF, GM-CSF, MIP-1, LIF, c-mpl ligand/thrombopoietin, erythropoietin, and flk2/flk3 ligand. The differentiation culture conditions will include at least two of the cytokines listed above, and may include several.

[0058] For example, if red blood cells are the desired mature blood product, at least erythropoietin will be added to the culture medium, and preferably SCGF, IL-1, IL-3, IL-6 and GMCSF all will be added to the culture medium, possibly with erythropoietin added later as a terminal differentiating factor. If platelets are the desired mature blood product, preferably SCGF, IL-1, IL-3, GMSCF and IL-11 will be

added to the culture medium. Figure 3 is a diagram showing the differentiation paths of blood cells with some of the cytokines known to promote differentiation of the various cell types. For example, the path for the differentiation of T cells requires that a TA cell be differentiated with IL-1 and IL-6, followed by differentiation with IL-1, IL-2 and IL-7, followed by differentiation with IL-2 and IL-4.

[0059] Alternatively to directing differentiation to a single cell type, if a single cell population is desired, the final product could be a mixed population and the cells could be separated using current cell separation techniques and procedures.

[0060] In one embodiment, the cytokines are contained in the media and replenished by media perfusion. Alternatively, the cytokines may be added separately, without media perfusion, as a concentrated solution through separate inlet ports in the differentiation bioreactor. When cytokines are added without perfusion, they will typically be added as a 10-100x solution in an amount equal to one-tenth to 1/100 of the volume in the bioreactors with fresh cytokines being added, for example, approximately every 2 to 4 days. Adding cytokines in this manner allows for progressive differentiation in the same differentiation bioreactor, as fresh concentrated cytokines also can be added separately in addition to cytokines in the perfused media.

[0061] In most aspects, suitable conditions for differentiation comprise culturing at 33 to 40°C, and preferably around 37°C. The initial oxygen concentration can be from about 20%, to about 1%, with 5% being approximately the normal physiological level. Lower oxygen concentrations have been associated with differentiation of cells to the erythrocyte lineage, so when specific cell products such as erythrocytes are desired the oxygen concentration can be kept as low as 1-5% oxygen, and preferably about 2-3% oxygen. Preferably, the cell concentration is kept at an optimum throughout differentiation.

[0062] Once differentiated, selection for the desired blood cell type can be performed by looking for cell surface markers. For example, T cells are known to have the markers CD2, 3, 4 and 8; B cells have CD10, 19 and 20; myeloid cells are positive for CD14, 15, 16 and 33; natural killer ("NK") cells are positive for CD2, 16

and 56; red blood cells are positive for glycophorin A; megakaryocytes have CD41; and mast cells, eosinophils and basophils are known to have markers such as CD38, CD71, and HLA-DR.

[0063] Returning to Figure 1, a single differentiation step is shown at step 130 and a single selection step is shown at 132 for the proliferated TA cells. However, it is contemplated that some differentiation schemes will require several, sequential differentiation and/or selection steps to achieve a homogeneous population of the desired mature cell. The possibility of such additional, sequential steps is indicated in Figure 1 as a combined step 140, comprising a differentiation step and a selection step. Alternatively, differentiation steps can be performed sequentially, without intervening selection steps. The varying number of steps 140 is indicated by n, where n is ≥ 1. Thus, such a multi-step differentiation process would involve a series of TA cells, all intermediaries, displaying progressive diminution of differentiation potential and increased cell maturity as the TA cells progress through the differentiation steps. Finally, at step 160, once the desired mature cells have been produced, they are transferred to an apparatus that adds a preservation agent and/or otherwise prepares and packages the cells for storage.

[0064] Preservation

[0065] The last step shown in Figure 1, step 160, is the preservation and packaging of differentiated cells. As described previously, the present invention envisions that to the extent possible, the system will be a closed one. That is, delivery of the mature blood cells from the differentiation bioreactor to one or more downstream chambers or apparatus, and optimally, packaging, will take place without human intervention in a closed, sterile system.

[0066] It is envisioned that in various embodiments of the present invention the downstream chambers or apparatus, will comprise one or more of the following: a chamber to wash and, if necessary, concentrate the cells or blood products; a chamber or apparatus to resuspend or perfuse the cells or blood products with a preservation or storage solution; and an apparatus to dispense and package the cells or blood products in sterile, transportable packaging. Such chambers may be

separate chambers or one or more function may be performed in the same chamber. In addition, an intervening cryopreservation step (and, hence, apparatus) also may also be added.

[0067] Preservation of blood cells can be accomplished by any method known in the art. For example, general protocols for the preservation and cryopreservation of biological products such as blood cells are disclosed in US Pat. Nos. 6,194,136 and 5,364,756 to Livesey, et al.; and 6,602,718 to Augello, et al. In addition, solutions and methods for the preservation of red blood cells are disclosed in US Pat. No. 4,386,069 to Estep.

[0068] Preservation of platelets is a more difficult problem and has, until recently, been met with limited success; however, method and preservative solutions are disclosed in US Pat. Nos. 5,622,867, 5,919614, and 6,211,669 to Livesey, et al., as well as recent reports regarding new methods from HyperBaric Systems, Inc. and Human Biosystems, Inc.

[0069] Packaging may be accomplished by any method or apparatus known in the art; optimally, without interruption of the sterile, closed environment. Packaging most often will involve apportioning the blood cells or blood products into sterile packaging and sealing of the packaging. An additional apparatus may be used to move the packaged product into the appropriate storage environment.

[0070] Genetic Manipulation

[0071] The blood cell products of the present invention may be modified by generating loss of function mutations by performing homologous recombination, targeted gene knockout or targeted integration in the hESCs used for culture. Such genetic manipulations may be desired particularly to delete or substitute cell surface antigens, such as histocompatibility antigens or blood group antigens.

[0072] Human Leukocyte Antigens (HLA) are part of the Major Histocompatibility Complex. The MHC comprises genes, including HLA, which are integral to normal function of the immune response. The essential role of the HLA lies in the control of self-recognition and defense against microorganisms. The HLA loci, by virtue of

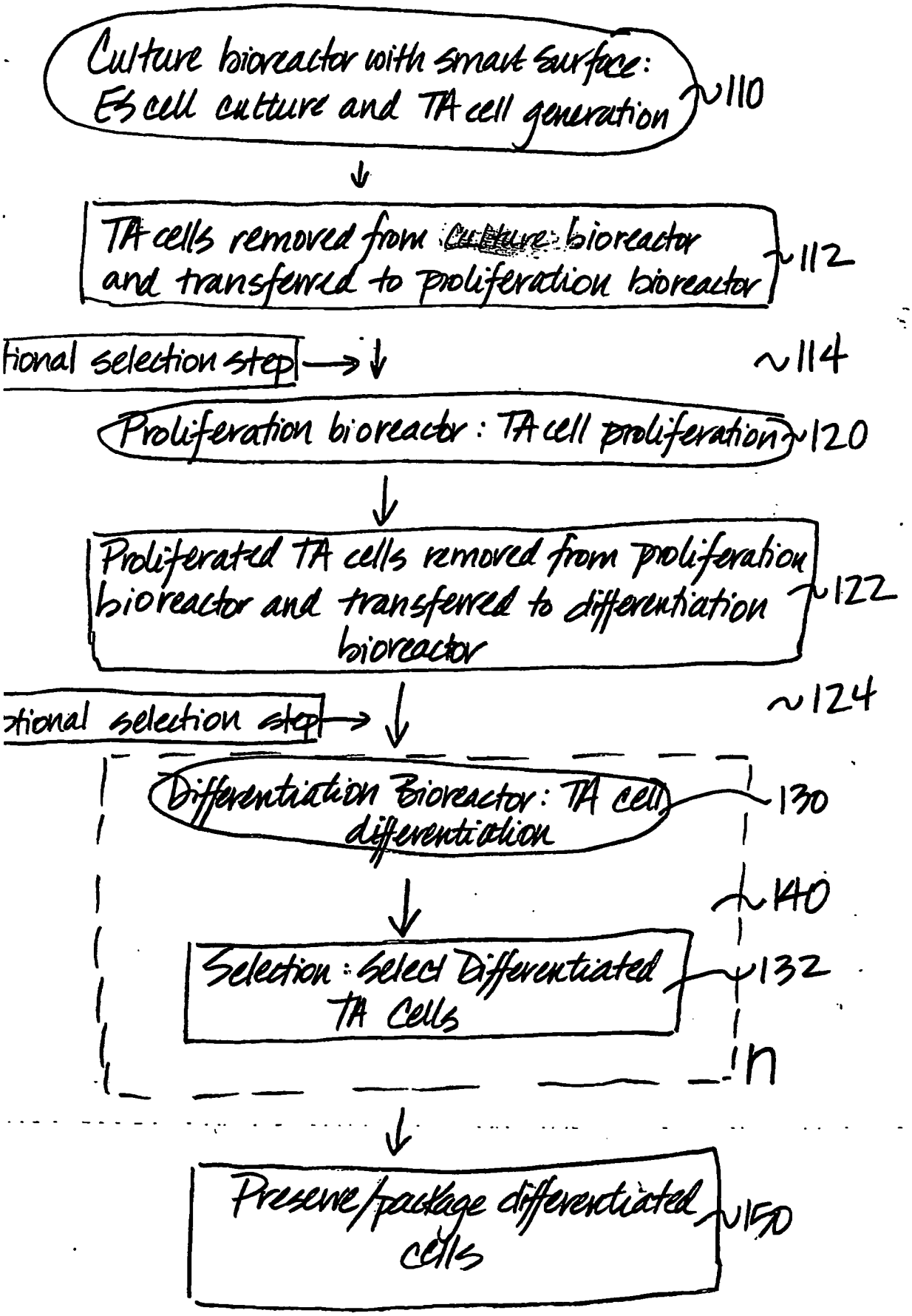
their extreme polymorphism, ensure that few individuals are HLA identical. HLA are recognized on all tissue cells of the body, including blood cells. Patients with intact immune systems who require multiple transfusions of whole blood, platelets or leukocyte concentrates will therefore usually develop antibodies to HLA antigens.

[0073] Techniques to generate recessive loss-of-function mutations in genes by are well known in the art (see, e.g., Capecchi et al., Science, 244: 1288 (1989); Hutchinson et al., Mutation Res., 299:211 (1993); Galli-Taliodoros et al., J. Immunnol. Meth., 181:1 (1995) (review); Robbins, Circ. Res., 73:3 (1993) (review); and Umans et al., J. Biol. Chem., 270:19777 (1995)). The techniques can be used to delete genes (null mutation) encoding for cell surface antigens, e.g., HLA histocompatibility and non-ABO blood group antigens on red blood cells, platelets, and other blood cells. For example, deletion of the beta-2-microglobulin gene will prevent expression of A, B and C antigens. In a similar manner, genes for the red blood cells antigens--Kell, Kidd, and Duffy--may, if desired, also be deleted. These techniques provide hESCs that can be differentiated into blood products that can be transfused into a patient without inducing antibodies which may limit further transfusions. After transfection, the cells are commonly selected by including a second gene construct (e.g., antibiotic resistance gene) that can be utilized in a positive-negative selection process.

[0074] While the present invention has been described with reference to specific embodiments, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, or process to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the invention.

ABSTRACT

The present invention provides methods for in vitro production of clinically useful quantities of differentiated human blood cells. In various embodiments of the present invention, immortal pluripotent cells, preferably universal donor cells, are produced from a cell production device. In a preferred embodiment, the device is a sequential series of bioreactors utilizing growth media containing specific combinations of maintenance-, proliferation- or differentiation-promoting factors that maintain, expand and promote the maturation and differentiation of the desired cell types. The immortal pluripotent cells can optionally be genetically modified so as to remove histocompatibility or blood group antigens.


Culture bioreactor with smart surface: ES cell culture and TA cell generation
110
TA cells removed from culture bioreactor and transferred to proliferation bioreactor
112
tional selection step
114
Proliferation bioreactor: TA cell proliferation
120
Proliferated TA cells removed from proliferation bioreactor and transferred to differentiation bioreactor
122
tional selection step
124
Differentiation Bioreactor: TA cell differentiation
130
140
Selection: Select Differentiated TA Cells
132
n
Preserve/package differentiated cells
150

100

FIG. 1

FIG. 2A

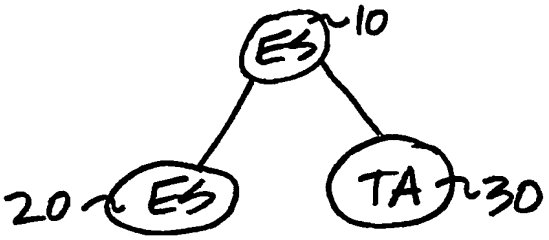
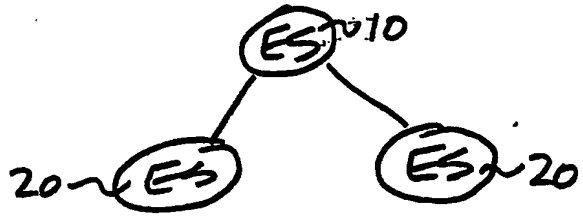


FIG. 2B



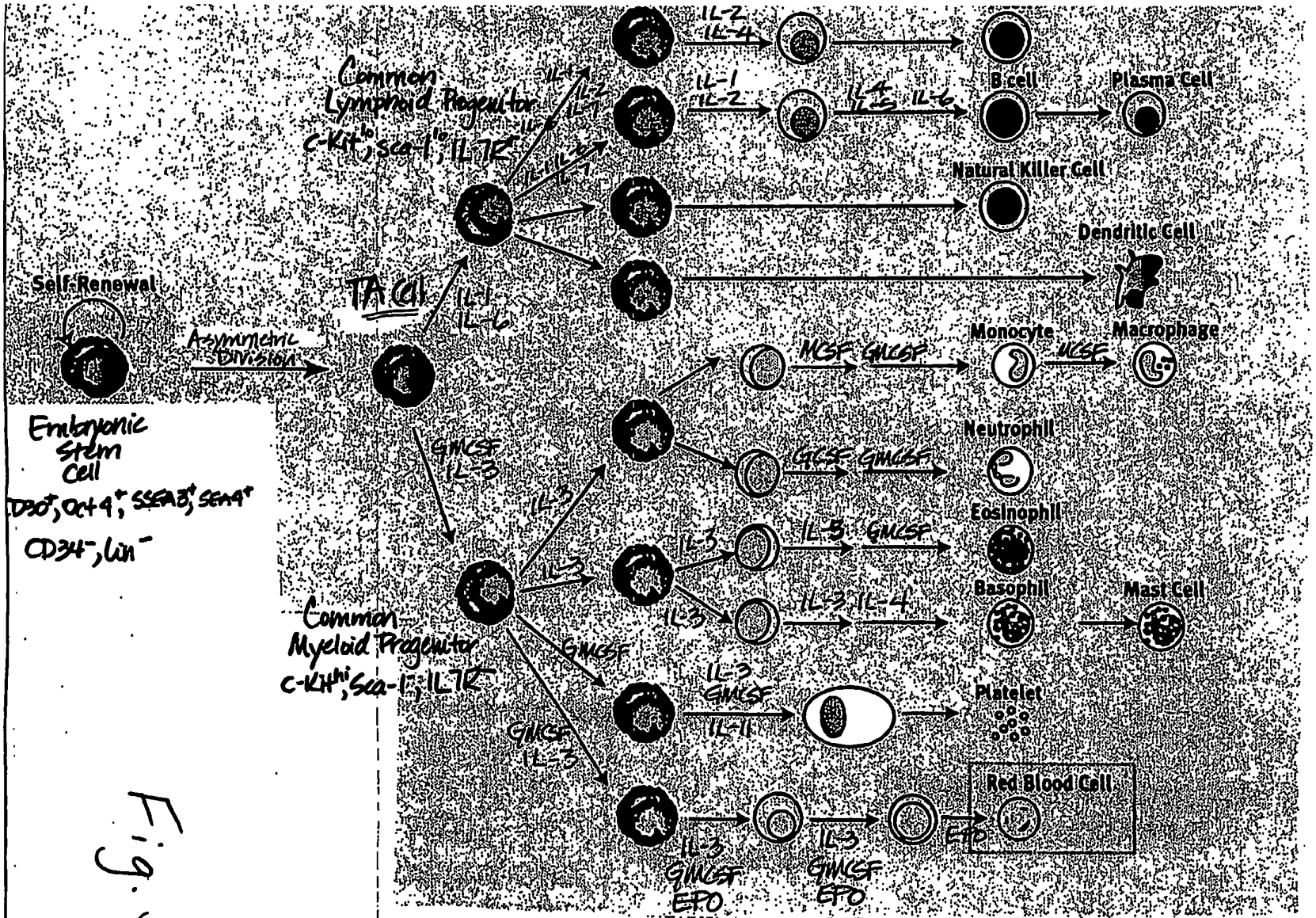

Embryonic Stem Cell
CD30+, Oct-4+, SSEA3+, SSEA4+
CD34-, Lin-
Self-Renewal
Asymmetric Division
TA Cell
Common Lymphoid Progenitor
c-Kit lo, Sca-1 lo, IL7R+
Common Myeloid Progenitor
c-Kit hi, Sca-1-, IL7R-
IL-1
IL-6
IL-2
IL-4
IL-1
IL-2
IL-4
IL-5
IL-6
B cell
Plasma Cell
Natural Killer Cell
Dendritic Cell
Monocyte
Macrophage
MCSF
GMCSF
IL-3
Neutrophil
Eosinophil
Basophil
Mast Cell
IL-5
IL-11
Platelet
Red Blood Cell
EPO

Fig. 3

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